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Am J Physiol Gastrointest Liver Physiol 285:G1162-G1170, 2003. First published 11 September 2003; doi:10.1152/ajpgi.00243.2003

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Total parenteral nutrition adversely affects gut barrier function in neonatal piglets

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Submitted 29 May 2003; accepted in final form 31 July 2003

Kansagra, Ketan, Barbara Stoll, Cheryl Rognerud, Harri Niinikoski, Ching-Nan Ou, Roger Harvey, and Douglas Burrin. Total parenteral nutrition adversely affects gut barrier function in neonatal piglets. *Am J Physiol Gastrointest Liver Physiol* 285: G1162–G1170, 2003. First published September 11, 2003; 10.1152/ajpgi.00243.2003.— Sepsis is the most common morbidity in preterm infants, who often receive total parenteral nutrition (TPN). We hypothesized that gut barrier function is compromised in TPN-fed compared with enterally fed newborn piglets (ENT pigs). Colostrum-deprived newborn pigs were implanted with jugular venous and bladder catheters under general anesthesia. Pigs were either administered TPN (n = 15) or fed formula (ENT pigs, n = 15). After 6 days, pigs were gavaged a solution of mannitol, lactulose, and polyethylene glycol 4000 (PEG 4000) and urine was collected for 24 h. At 7 days, small bowel samples were assayed for myeloperoxidase activity, morphometry, and tight junction protein abundance. Intestinal contents and peripheral organ sites were cultured for bacteria. Urinary recovery (%dose) of mannitol (53 vs. 68) was lower, whereas that of lactulose (2.93 vs. 0.18) and PEG 4000 (12.78 vs. 0.96) were higher in TPN vs. ENT pigs, respectively ($P < 0.05$). Incidence of translocation was similar in TPN and ENT pigs. Myeloperoxidase activity was increased in TPN vs. ENT pigs in the jejunum ($P < 0.001$) and was weakly correlated with lactulose (R^2 = 0.32) and PEG 4000 (R^2 = 0.38) recovery. Goblet cell counts did not change, but intraepithelial lymphocyte numbers decreased with TPN. Only claudin-1 protein abundance was increased in the TPN group. We conclude that TPN is associated with impairment of neonatal gut barrier function as measured by permeability but not translocation.

neonates; permeability; tight junctions; intraepithelial lymphocytes; goblet cells

LATE-ONSET SEPSIS is the number one morbidity in neonatal infants. Many factors have been implicated in the increased incidence of infection in preterm infants, including total parenteral nutrition (TPN) (32). Risk factors associated with TPN that may increase the incidence of late-onset sepsis in neonates are multifactorial and include the presence of central venous catheters, very low birth weight (<1,500 g), and mechanical ventilation. However, TPN results in deprivation of luminal nutrition that also adversely affects the mucosal integrity and may compromise barrier function of the neonatal gut, resulting in a phenomenon described as "gut-derived" sepsis. Gut-derived sepsis results, in part, from deterioration in the mucosal epithelial barrier and subsequent translocation of luminal bacteria and toxins into the blood (4).

Studies have shown that gut mucosal atrophy occurs in the absence of total or partial (<60% total caloric intake) enteral nutrition in neonatal and adult animals (6, 33) and, to a lesser extent, in human infants (44). TPN-induced mucosal atrophy is characterized by morphological changes, such as reduced villus height and mucosal surface area, which are secondary to decreased crypt cell proliferation and protein synthesis and to increased apoptosis (7). TPN not only reduces the population of enterocytes but also has been associated with expansion of mucosal goblet cells (GCs) and some lymphocyte subtypes (11, 30). Furthermore, newborn (11, 19) and adult (17) animal studies indicate that TPN-induced activation of lymphocytes may induce mucosal inflammation on the basis of increased ICAM-1 expression and myeloperoxidase activity. Studies in TPN-fed mice demonstrated that increased expression of the proinflammatory cytokine INF-γ in activated intraepithelial lymphocytes (IELs) is linked to increased apoptosis and may contribute to the breakdown of epithelial barrier integrity (26, 63).

This breakdown in barrier integrity during TPN administration has been evaluated experimentally via the assessment of bacterial translocation, defined as the passage of enteric bacteria to the mesenteric lymph nodes and other extranodal sites. Alverdy et al. (1) found that TPN was associated with increased bacterial translocation in adult rats. The increase in bacterial translocation has been associated with higher rates of sepsis in human neonates/infants (42) and adult surgery patients (41) receiving TPN.

TPN appears to negatively impact another measure of gut barrier function, paracellular permeability. Per-

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meability has long been considered an indicator of gut dysfunction in many disease states, from trauma and burns, to malnutrition (59), to inflammatory bowel disease (IBD) (43). Cell monolayer experiments have shown increased permeability when exposed to INF-γ (35). A study in adult rats noted a concomitant increase in in vitro intestinal permeability as well as bacterial translocation in TPN-fed animals (12). Cell culture experiments and adult clinical studies have also supported this finding of increased permeability in critically ill patients on TPN (20, 35).

As far as we are aware, a cellular mechanism for the increase in intestinal permeability during TPN has not been completely elucidated, especially in the newborn. A potential explanation can be a breakdown in tight junction (TJ) integrity. TJs are a group of proteins intimately involved in intercellular adhesion. Patients with IBD, known to have increased permeability, have also been found to have downregulation of TJ proteins, particularly occludin (29).

These various observations have not been evaluated simultaneously and prospectively in an in vivo newborn model. Therefore, we hypothesize that TPN-fed neonatal piglets have compromised gut barrier function as measured by bacterial translocation and paracellular permeability, and this deterioration is related to increased gut inflammation and impaired TJ integrity.

MATERIALS AND METHODS

Animals

The study protocol was approved by the Animal Care and Use Committee of Baylor College of Medicine and was conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" [DHEW Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205]. For the study, pregnant, crossbred sows (Large White × Hampshire × Duroc) were obtained from the Texas Department of Criminal Justice (Huntsville, Texas) ~1 wk before estimated date of delivery. They were housed, fed, and kept under surveillance in the animal facility at the Children's Nutrition Research Center (Houston, TX) until delivery. The sows delivered the piglets vaginally. Remote video cameras were used to monitor the sows around the clock, allowing collection of the piglets before suckling. The newborn piglets were kept in heated cages and fed only water until surgery.

Study Design

Surgical procedure. Figure 1 illustrates an overview of the study. Preoperatively, each animal received only one systemic dose of enrofloxacin (2.5 mg/kg; Bayer, Shawnee Mission, KS). We gave only one dose to prevent confounding of culture data, although previous studies indicate that systemic antibiotics affect intestinal microflora minimally (23, 24, 31). Piglets were surgically catheterized within 12 h of birth under isoflurane general anesthesia. Catheters were inserted into the jugular vein, carotid artery, and bladder. Silastic vascular catheters were inserted via a cutdown procedure as previously described (49). Bladder catheter insertion initially involved a midline (females) or para-abdominal (males) vertical incision caudal to the umbilicus. The peritoneal cavity was entered, and the bladder was isolated. A purse-string monofilamentous suture was placed in the anterior wall of the bladder. A 16- or 18-gauge needle was then used to create a vesicotomy. A Silastic catheter was inserted into the vesicotomy and secured with the purse-string suture. A second stitch was used to anchor the catheter to the abdominal wall. The catheter was then tunneled subcutaneously and brought out through the skin dorsally. A two-layered closure was utilized to close the abdominal incision. Bactericidal ointment was placed over the incision and covered with gauze. Catheters were secured in a jacket that was attached to a tether. The tether allowed safe and secure administration of TPN to the piglets once they were in their cages. Postoperatively, the piglets received one dose of analgesic (0.1 mg/kg butorphenol tartrate; Fort Dodge Labs, Fort Dodge, IA).

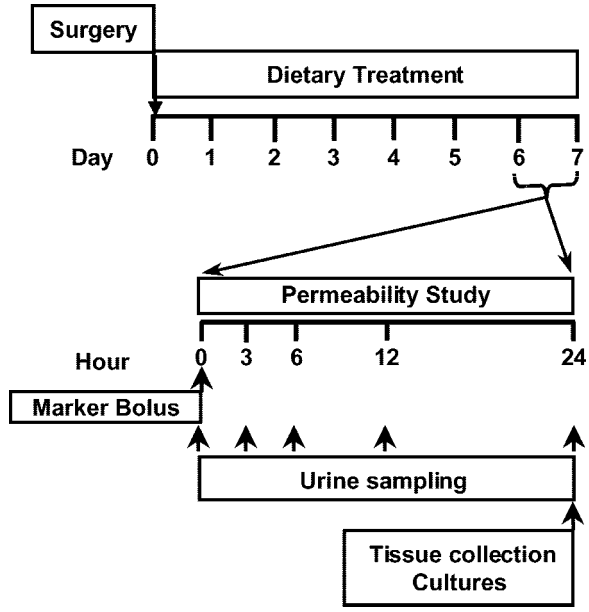

Fig. 1. Piglets received enteral nutrition (ENT) or parenteral nutrition (TPN) for 7 days with intestinal permeability measurements made during the last 24 h before tissue sampling.

Nutritional protocol. Pigs were divided into two equal groups, one of which was placed on 240 ml·kg^{-1}·day^{-1} TPN [25 g·kg^{-1}·day^{-1} glucose, 13 g·kg^{-1}·day^{-1} protein (amino acids), and 5 g·kg^{-1}·day^{-1} lipid (Intralipid 20%; Fresenius Kabi, Bad Hamburg, Germany)], and the other group fed liquid milk replacer [enterally fed pigs (ENT pigs) 240 ml·kg^{-1}·day^{-1} Advance Liqui-Wean; Milk Specialties, Dundee, IL; mixed 1:5 powder/water] for 6 days. The enteral feedings were divided evenly 5–6 times per day. The liquid milk replacer did not contain antibiotics. The nutritional support was continued until euthanasia. Total caloric intake was not different between the two groups (~840 kJ·kg^{-1}·day^{-1}).

Outcome Measurements

Intestinal permeability. On *day 6*, both groups received an intragastric bolus of three permeability marker molecules of mannitol (50 mg/kg; Sigma, St. Louis, MO), lactulose (500 mg/kg; Sigma), and polyethylene glycol 4000 (PEG 4000, 3 g/kg; Fluka Chemika, Steinheim, Switzerland) at *time 0*. These markers were dissolved in sterile water to make a 10 ml/kg solution, which was filtered through a 0.2-μm filter (Nalgene Filtration Products, Nalge Nunc International,

Rochester, NY) before administration. Mannitol is absorbed via the transcellular route, whereas lactulose and PEG are markers of paracellular permeability. We administered mannitol to control for a potential concern of delayed gastric emptying in the TPN piglets. Baseline urine samples were obtained before administering the marker solution. Subsequent urine samples were collected at 3, 6, 12, and 24 h. Specifically, the urine was collected via the implanted catheters, which were allowed to empty by gravity into a beaker kept on the floor at a level below that of the pig. Total collection of the urine was obtained during each interval.

Urine was analyzed as previously described (8, 46). In brief, urine was centrifuged in a 1.5-ml microcentrifuge tube at 3,000 rpm for 5 min. A 20-μl aliquot of the urine was injected onto an Aminex HPX 87C 300 7.8-mm cation-exchange column protected with a precolumn of Deashing system (BioRad Laboratories, Hercules, CA) and eluted with degassed pure water at a flow rate of 0.6 ml/min at 85°C. The column effluent was monitored with a differential refractometer (Millipore, Medford, MA) with the sensitivity setting at 128, scale factor at 25, and internal temperature at 50°C. The column was calibrated by using PEG 4000, lactulose, and mannitol as standards. The coefficient of variation with this method is ≤5%.

Bacterial translocation. At the end of the collection period, the piglets were euthanized with an intravenous injection of pentobarbital sodium (50 mg/kg) and sodium phenytoin (5 mg/kg; Beutanasia-D; Schering Plough Animal Health, Union, NJ). Subsequently, under sterile conditions, samples of blood (via direct cardiac puncture), liver, spleen, mesenteric lymph nodes, and intestinal lumens were cultured for aerobic and anaerobic bacteria. Anaerobic transport medium was used for transport of anaerobic samples (Anaerobe Systems, Morgan Hill, CA). Tissue samples were diluted 1:1 with PBS before plating. Blood was streaked directly onto plates, and gut contents were diluted from log 10^{-2} to log 10^{-5} before plating. Restrictive media used for bacterial isolations were Anaerobic Brucella blood agar for *Clostridium perfringens* (Anaerobe Systems, Morgan Hill, CA); *Bacterioides* bile esculin agar for *Bacterioides* (Anaerobe Systems); and cycloserine-cefoxitin fructose agar for *C. difficile* (Anaerobe Systems). Plates were incubated under anaerobic conditions for ≤4 days at 37°C. Media for aerobic isolation isolations included Rogosa SL Agar for lactobacilli; MacConkey agar for *Escherichia coli*, *Klebsiella*, and *Enterobacter*; *enterococcus* agar for *Enterococcus*; and mannitol salt agar for *Staphylococcus* (all from Difco, Detroit, MI). Plates were incubated at 37°C for 48 h under aerobic conditions. Further identification (species) of isolates was done by API biochemical test kits (bioMerieux, Hazelwood, MO).

Myeloperoxidase activity. As an indicator of inflammation, we assayed tissue myeloperoxidase activity by using a modification of the method by Suzuki et al. (51) for use in a 96-well microplate reader (Molecular Devices, Sunnyvale, CA). Full-thickness tissue samples were homogenized at 20,000 *g* twice, with the second centrifugation step being preceded by the addition of a detergent, hexadecyltrimethylammonium bromide, to disrupt the cell membranes, as previously described (28). Activity was on the basis of a standard curve of human myeloperoxidase standards (Sigma-Aldrich, St. Louis, MO) with dilutions from 1:4 to 1:256.

Intestinal morphology and cell counts. The small bowel was measured for wet weight and length after cold saline rinse to remove intestinal contents on collection. Sections were placed in 10% formalin for histology. Further assessment of villous height and area and crypt depth were made on 5-μm hematoxylin and eosin-stained sections by using standard light microscopy (Axiophot; Carl Zeiss, Werk Göttingen, Germany) and NIH Image software, version 1.60 (National Institutes of Health, Bethesda, MD). Ten well-formed villi from each sample were used for analysis. A blinded observer counted IELs and GC counts morphologically on hematoxylin and eosin-stained sections by using a high-power objective.

TJ protein abundance. One gram of pulverized frozen tissue was homogenized in 2 ml buffer (in mM: 50 HEPES, 150 NaCl, 5 NaEDTA) and protease inhibitor cocktail (2 mg/ml bacitracin, 348 mg/l PMSF, 18 mg/50 ml buffer), 25 μg/ml leupeptin (1.25 mg/50 ml buffer), and aprotinin (25 μg/ml; 1.25 mg/50 ml buffer). Homogenate was centrifuged at 8,000 *g* (10,000 rpm) for 10 min at 4°C. Supernatant was removed and centrifuged at 100,000 *g* (39,000 rpm) for 30–45 min at 4°C. Supernatant was discarded, and membrane pellet was resuspended in buffer plus 1% Triton X-100 (2 ml). The pellet was homogenized and solublized for 24 h with gentle rocking at 4°C.

Equal protein amounts were loaded into SDS-PAGE gels and run at 100 V for 60 min and then transferred to nitrocellulose (100 V for 60 min). Membrane was blocked with 3% nonfat dry milk in Tris-buffered saline (TBS). Rabbit polyclonal antibody (Zymed, South San Francisco, CA) was used for claudins 1 and 2 in concentrations of 1:250 and 1:500, respectively. For occludin and zonula occludens-1 (ZO-1), rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were also used in concentrations of 1:500 and 1:400, respectively. After being washed with TBS-Tween 20 buffer, the membrane was incubated with a secondary antibody (anti-rabbit IgG conjugated with biotin; Santa-Cruz Biotechnology) at room temperature for 1 h. The membrane was then enhanced with Neutravidin-horseradish peroxidase (Pierce) in the nonfat dry milk-TBS solution for 1 h at room temperature and allowed to react with horseradish peroxidase substrate (SuperSignal; Pierce) for 5 min. Finally, the membrane was exposed to X-ray film for 30–180 s, and the image was scanned and quantified by ImageQuant 5.0 software (Molecular Dynamics, Amersham Biosciences, Sunnyvale, CA).

Statistical Analyses

One-way ANOVA was used for all normally distributed data comparisons and χ^2 was used only for nonparametric data. Pearson correlations were used when indicated. All data shown are means ± SE, unless otherwise stated. Statistical significance was determined to be $P < 0.05$. Statistical calculations were carried out by using Minitab version 13.32 (Minitab, State College, PA).

RESULTS

Pig Body Growth and Urine Output

There was no difference in birth weight (1.56 vs. 1.59 kg) or weight gain (44.8 vs. 42.3 $g \cdot kg^{-1} \cdot day^{-1}$) in TPN vs. ENT pigs during the study period. Urine output ($ml \cdot kg^{-1} \cdot day^{-1}$) as measured during the final 24 h of the study was also not different (81.94 TPN vs. 84.75 ENT).

Gut Morphometry and Cell Counts

In the jejunum, wet weight (g/kg body wt), villus height (μm), and area (μm^2) were significantly reduced in the TPN group when compared with the ENT pigs.

Table 1. *Jejunal and ileal gut morphometry*

	Jejunum			Ileum		
	TPN	ENT	*P* Value	TPN	ENT	*P* Value
Mass, g/kg body wt	10.92 ± .78	16.75 ± .80	<0.001	12.24 ± 0.62	18.51 ± 0.69	<0.001
Villus height, μm	542 ± 95	975 ± 51	0.003	617 ± 73	722 ± 53	0.273
Villus area, 10^3 μm^2	35.8 ± 8	81.6 ± 5	0.001	47.3 ± 8	56.8 ± 7	0.386
Crypt depth, μm	93.8 ± 5.2	107.9 ± 5.3	0.085	92.9 ± 5.2	103.7 ± 4.0	0.133
Protein content, mg	4094 ± 1182	7868 ± 838.3	0.001	2905 ± 327.7	6489 ± 666.3	<0.001
DNA content, mg	151 ± 12	195 ± 17	0.043	136 ± 8	239 ± 20	<0.001

Values are means ± SE; n = 15 total pigs in study used to determine mass; n = 6 samples each used to determine villus height and area; n = 12 samples each were used to determine crypt depth, protein content, and DNA content. *P* values were determined by one-way ANOVA.

Total protein content (mg) and DNA content (mg) expressed in absolute amounts were decreased in the TPN piglets. Crypt depth (μm) was similar in both treatment groups (Tables 1 and 2).

Ileal measurements follow a similar trend. Wet weight, protein, and DNA contents were less in the TPN vs. ENT groups. Unlike the jejunum, no difference was noted with regards to villus height and area and crypt depth. Cell counts were corrected for villus size differences between treatment groups. IELs showed a marked reduction in number when the animals received TPN; however, there seemed to be no effect of enteral nutrition on the number of GCs per villus.

Intestinal Permeability

As a measure of paracellular permeability, we assessed pooled 24-h urinary recovery of markers expressed as a percentage of the dose recovered. Twenty-four-hour collections were used to obtain adequate marker recovery. We found 75 and 88% of the total recovered amount of mannitol within 12 h in the TPN and ENT groups, implying that collecting urine beyond 24 h would have resulted in insignificant changes to the data. Lactulose recovery was significantly greater at 2.931 ± 0.67 vs. 0.179 ± 0.11% between TPN and ENT groups, respectively. Similarly, PEG 4000 recovery was 12.78 ± 4.11 vs. 0.96 ± 0.24% between the same groups. To ensure that pre- and postintestinal factors did not affect recovery of the paracellular markers, we used mannitol absorbed via the transcellular route as a controlling factor, and recovery was notably reduced in the TPN group compared with the ENT group (52.99 ± 3.86 vs. 68.35 ± 3.94%, respectively; Fig. 2). Mannitol is often used with lactulose to correct for the latter's recovery, because the two markers are not metabolized in the small bowel, empty similarly from the stomach, and are cleared in the same manner from the kidneys (5). When lactulose/mannitol ratios were used to corroborate recovery data, the results were no different (0.0504 ± 0.0100 TPN vs. 0.0023 ± 0.0014 ENT).

Table 2. *Jejunal IEL and GC counts*

	TPN	ENT	*P* Value
Goblet cells/villus	9.14 ± 1.53	7.28 ± 1.7	0.272
IELs/villus	3.72 ± 0.79	11.1 ± 1.2	<0.001

Values are means ± SE; n = 6 samples. Values were corrected for villus size. GC, goblet cells; IELs, intraepithelial lymphocytes. *P* values were determined by one-way ANOVA.

Intestinal Inflammation

Myeloperoxidase activity expressed as millunits of activity per milligram of protein was used as an indirect indicator of inflammation. One unit of activity equals 1 μM of substrate catalyzed in 1 min at 25°C. Our findings showed a significant increase in enzyme activity in the proximal jejunum (4.63 ± 0.47 vs. 2.16 ± 0.39 mU/mg protein) and a trend toward increased activity in the distal ileum [5.66 ± 0.58 vs. 4.03 ± 0.66 mU/mg protein (P = 0.08)] of the TPN group compared with controls (Fig. 3).

Weak but significant correlation estimates were found for myeloperoxidase activity and recovery of lactulose and PEG 4000 [R^2 = 0.32 (P = 0.003) and R^2 = 0.38 (P = 0.003), respectively] when the animals were assessed collectively. No such correlation was found for bacterial translocation and either myeloperoxidase activity or permeability.

Bacterial Translocation

We assessed translocation of bacteria from the lumen of the jejunum, ileum, or cecum to peripheral sites, which included liver, spleen, mesenteric lymph nodes, and peripheral blood. We defined a translocation event as the presence of the same bacterium in both the lumen and in one or more peripheral sites. On the basis of our definition, we found no difference in bacterial translocation between TPN and ENT groups (62 vs. 69%). Also, we noted that a larger variety of

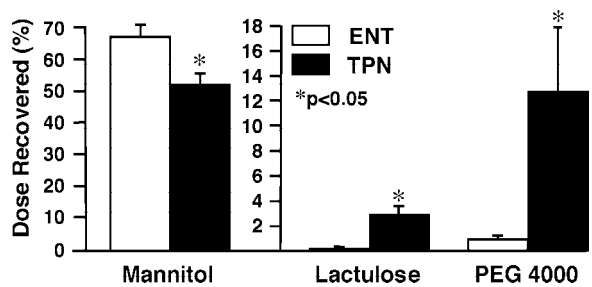

Fig. 2. Urinary recovery of permeability markers (%dose administered) in 24 h in ENT vs. TPN piglets were determined via HPLC. Values are means ± SE.

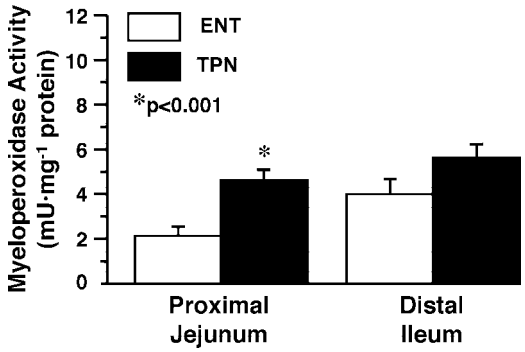

Fig. 3. Myeloperoxidase activity (milliunits activity per milligram protein) in the proximal and distal small bowel of ENT vs. TPN piglets was increased in the latter group. Values are means ± SE.

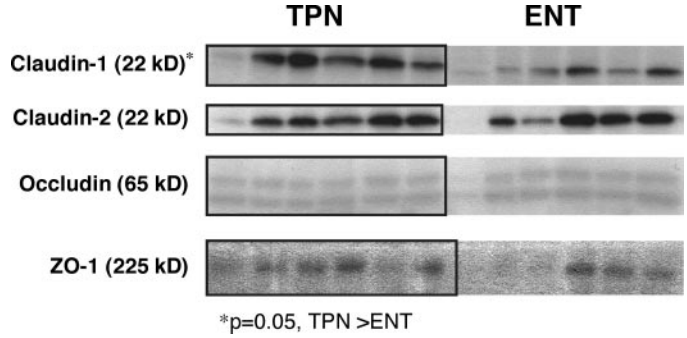

Fig. 4. Densitometric measurement of TJ protein abundance in jejunal tissues of ENT and TPN groups. Claudin-1 (22 kDa) abundance was significantly increased in the TPN group, but the remainder of tested TJ proteins did not change.

organisms translocated in the ENT group, although the prevalence of translocation did not differ (Table 3).

TJ Protein Abundance

Western blot analysis of gut mucosal samples, evaluated by densitometry, revealed only an increased expression of claudin-1 in the TPN group when compared with ENT pigs. We found no difference in the amounts of claudin-2, occludin, and ZO-1 as assessed by Western blotting (Fig. 4).

DISCUSSION

TPN has been a life-saving therapy used in hospitalized patients, especially those admitted to neonatal intensive care units. Newborns admitted to neonatal intensive care units and receiving TPN are at greater risk for late-onset sepsis. TPN can increase the risk for infection in many ways, but we focused on its effects on gut barrier function. Despite being clinically beneficial, TPN is the deprivation of enteral nutrition and is associated with intestinal changes in structure and function. Adult animal studies have demonstrated increases in bacterial translocation and intestinal permeability, but the degree to which these are altered in the newborn model has not been described. Therefore, we hypothesized that barrier function would be compromised in the TPN-fed piglets. Interestingly, we did see that gut barrier function was diminished in the TPN group but only as measured by intestinal permeability not bacterial translocation.

Table 3. *Prevalence of bacterial translocation and frequency of individual translocating bacterial species*

	Frequency of positive, *n*	
	TPN, Total Events = 62%*	ENT, Total Events = 69%*
Enterococcus	7/13	7/13
Klebsiella	NT	5/13
Staphylococcus	NT	2/13
Enterobacter	NT	1/13
Pediococcus	NT	1/13
Clostridium	1/13	NT

One translocation event was defined as one or more bacterial species found in peripheral and intestinal sites of the same animal. Bacterial species are listed in order of decreasing frequency. They are for descriptive purposes only and individual comparisons have not been made. *χ^2 analysis for assessing difference in bacterial translocation prevalence. NT, not translocating.

Mucosal Atrophy

Consistent with many previous studies (6, 33), we have shown that TPN (i.e., the lack of enteral nutrition) leads to gut atrophy, specifically mucosal atrophy. The present study showed notable decreases in jejunal mass (34.8%), villus height (44.4%), and villus area (56.1%) of TPN-fed piglets compared with controls. However, in the ileum, only tissue mass (33.9%), protein, and DNA content were reduced by TPN, whereas villus height and area were unaffected. These findings are not necessarily novel and highlight the fact that the proximal mucosa is more susceptible to lack of ENT nutrients than the distal gut (33).

Intestinal Permeability

This study was designed to examine in breadth neonatal gut barrier function. Although there is no single "ideal" test for quantifying barrier function, measures of intestinal permeability have been used frequently by many investigators. Intestinal permeability generally implies paracellular permeability and is measured in vitro via ion flux or electrical resistance/voltage changes or or in vivo via excretion of inert markers into the urine.

In the present study, both paracellular permeability and bacterial translocation (see *Bacterial Translocation*) were measured. As expected, we found an increased urinary recovery of the paracellular markers, lactulose, and PEG 4000 in piglets fed TPN. Marker recovery with the enteral nutrition-fed gut was similar to that of a 4-wk-old pig, having very little marker being absorbed from the bowel lumen (60). These previous studies in pigs and those in humans (46) have shown that intestinal permeability is high at birth and declines with advancing postnatal age. The relative amount of PEG 4000 recovered compared with lactulose was greater, which may be due to a larger dose of PEG 4000 and the molecular structure of PEG 4000.

Although having an average molecular weight of 4,000 kDa, the shape of a PEG 4000 molecule is linear compared with a lactulose molecule, which is bulky (5, 50). Therefore, the ability for a narrow PEG molecule to pass through openings in the TJs may be greater than lactulose. Nonetheless, the pattern of recovery for the two markers was similar. We also used mannitol, a transcellular marker, to confirm paracellular marker recovery accuracy. The ratio with which they are recovered should not change, and if a change is noted, it is due to alterations in lactulose recovery (3, 58).

Cellular mechanisms involved in the increased permeability during TPN have not been completely established; however, gut inflammation may play a role. In rodents, Fukatsu et al. (17) found that myeloperoxidase activity was increased in animals fed TPN. Furthermore, they found an increase in intercellular adhesion molecule-1 expression in the intestinal tissue of the animals fed TPN. Given that in the proximal small bowel we saw a significant increase in myeloperoxidase activity, which can be a surrogate for polymorphonuclear leukocyte (PMN) infiltration or inflammation, we looked for a possible link to the increased permeability. Our correlation estimates showed a significant positive relationship between the recovery of lactulose and PEG 4000, explaining 32 and 38% of the change in marker recovery. Moreover, the increase in inflammation proximally was inversely related to mucosal villus height and area.

IBD is a clinical disorder including Crohn's disease and ulcerative colitis, in which PMNs infiltrate into the intestinal mucosa and lead to clinical manifestations (21, 25). A portion of the pathophysiology involving the increased permeability seen in IBD may relate to alterations in the TJ and PMN infiltration (29). TJs are a complex of proteins forming essential structures that function to keep mucosal domains separate (e.g., apical and basolateral areas of enterocytes) and also to create a barrier to the passage of paracellular solutes. They are made up of intracellular scaffolding components intimately linked to the cell membrane and also consist of intercellular strands or fibrils that actually span the space between cells. ZO-1 is a scaffolding protein, and occludin and the claudins are strand proteins (52, 53). Thus we examined the abundance of TJ proteins to help clarify the molecular mechanism of increased paracellular permeability in neonates fed TPN.

As seen in Fig. 4, abundance was notably increased for the TJ protein claudin-1 in the TPN group. This was an unexpected finding, given that paracellular permeability was increased in that group. Studies have demonstrated that transepithelial resistance (a measure of epithelial integrity) increases with increasing strand number, but exceptions have been noted in cell culture models (9, 10, 48). Investigators have suggested that TJ fibrils are heteropolymeric, with differing claudin superfamily subtypes combining to form varying degrees of "adhesiveness" (54). Also mentioned, TJ strands are repeatedly broken and reannealed to give the junction a certain tightness (55). Because so little is known about the molecular regulatory mechanisms of TJs, it would be difficult to speculate on the nature of the current finding. The remaining proteins, although demonstrating no difference in abundance, may change their localization. Thus electron microscopy and confocal microscopy may be required to determine changes in the localization of TJ integral proteins in the face of TPN feeding (16).

The underlying cellular mechanisms linking mucosal inflammation to epithelial permeability and TJ alterations are not fully established. However, recent studies have demonstrated a potential connection between IEL function and loss of mucosal integrity and permeability in TPN-fed mice. This study quantified IELs in an attempt to assess the effects of lack of enteral nutrition on their numbers. IELs are the first immune cells to be exposed to foreign antigens and have the potential to be critical in host defense. A previous experiment in neonatal piglets demonstrated increased cytotoxic T lymphocytes in the lamina propria as well as increased GCs, implying that inflammation may play a role in the pathogenesis of TPN-associated mucosal changes (19). In addition, Li et al. (34) found that lymphocytes play an important role in the regulation of mucosal immunity in a mouse model. Also, they noted that a change in the ratio of $CD4^+$/$CD8^+$ T cells in TPN-fed mice are related to decreases in secretory IgA, a key player in the prevention of bacterial adherence to epithelium.

However, recent studies in mice (30) have shown that TPN decreases IEL numbers. Moreover, these changes in IELs increased INF-γ, which has been shown to decrease TJ integrity (27). Previous cell culture experiments confirm this loss of integrity due to INF-γ and other cytokines through alterations in the TJ (36, 57, 64). Kiristioglu et al. (26) also found that the $CD8\alpha\beta^+$ IELs, responsible for IEL proliferative activity, declined with TPN administration. Moreover, the $CD44^+$ IELs, which are mature lymphocytes, also decrease with TPN administration. These findings support the current finding of decreased IELs in the TPN group and may even help explain the increased apoptosis of enterocytes associated with TPN or the lack of ENT nutrients (62).

Bacterial Translocation

Another commonly used assessment of barrier function is bacterial translocation, which is the passage of enteric bacteria from the bowel lumen to peripheral sites. This is generally reported as positive cultures from peripheral sites containing "enteric" bacteria with the reasonable supposition that the bacteria were indeed from the gut (41). It is important to note that in the present study we verified positive peripheral cultures with concurrent cultures from the gut lumen. Translocation has been described as early as the 1960s and was reported to be increased in adults receiving TPN (1, 61). In addition, a study of infants on TPN (42) demonstrated an association between translocated gut bacteria and blood culture-confirmed septicemia and concluded that sepsis in infants receiving TPN may be

a gut-related phenomenon. Another report found increased postoperative sepsis in adult surgical patients showing evidence of bacterial translocation (41% BT vs. 14% non-BT; $P < 0.001$) (41).

Investigators have found that the primary site for translocation, in adult guinea pigs, is the small bowel (18). This finding was corroborated with a significant association of small intestinal bacterial colonization and translocation in newborn rabbits (56). Unlike the former study, the latter experiment was not designed to test TPN vs. enteral nutrition. The authors also speculated that this may explain why sepsis and necrotizing enterocolitis, an inflammatory condition strongly associated with prematurity, occur after neonates are 3–5 days of age, once the small bowel has been populated.

Uniquely evident in this experiment is that TPN, despite being associated with increased permeability and gut atrophy, did not increase translocation in the newborn piglet. Compared with the changes previously noted, there does not appear to be a correlation with the incidence of bacterial translocation. One group attempted to correlate bacterial translocation with plasma levels of PEG 4000 (2). Although the authors attempted to provide a practical method to assess translocation, the limitations of the study prevented extrapolation to widespread use. The assumption was made that increases in macromolecule permeability indicate a greater propensity for translocation. This presumption overlooked the fact that the mechanisms responsible for paracellular permeability and bacterial translocation are most likely not the same. Adult animal and human studies and the current newborn model confirm that these measures of gut barrier function are not strongly related (22, 40). Furthermore, despite finding slightly over half the piglets with translocation in both groups, we did not observe any clinical signs of sepsis.

Another factor that may affect bacterial translocation is the innate immune function of the GC. As part of the innate immune system, GC alterations may impact on gut barrier integrity. GC mucin production occurs via two processes, simple and compound exocytosis, describing baseline and enhanced secretion in response to stimuli, respectively (13). Many factors can impact on GC number and mucin production, including changes in diet and local microflora (15, 45). Also, TPN administration has been shown to increase jejunal sulfomucin-producing GCs, a finding that is consistent with the GC population of the fetal intestine and indicative of a more bacteriostatic mucus (11). In contrast to the previous study, we found no difference in the number of GCs between enteral nutrition- and TPN-fed piglets. A possible reason for this discrepancy is because we did not specifically identify GC type on the basis of mucin composition. Perhaps specific stains will show that the sulfomucin numbers did change despite total GC numbers remaining constant. However, we did find that *Clostridium* species was one of only two bacteria found to translocate in the TPN group. This finding is consistent with recent reports from Deplancke et al. (14) showing that TPN is associated with selective colonization of mucolytic bacteria, namely the *Clostridium* species. A link between *Clostridium* and epithelial dysfunction also has been demonstrated through alteration of the TJ (39).

Perspectives

In summary, TPN administration in newborn piglets led to mucosal atrophy and increased paracellular permeability and decreased IELs, but bacterial translocation and GCs were not different between the two groups as was TJ abundance. We propose that TPN or the lack of enteral nutrition leads to impaired gut barrier function as assessed by intestinal permeability and that bacterial translocation and permeability are not interchangeable entities in the measurement of gut integrity. Investigators had previously shown in preterm neonates that early feeding with human milk reduces gut permeability (46). Although the importance of human milk to premature infants cannot be overstated, not all babies are able to receive mother's milk for a variety of reasons. Therefore, beginning enteral feeding as early as possible may enhance gut barrier function, in addition to other functional improvements as previously described (38, 37, 47). Although TPN is an important development in the care of newborns, enteral nutrition appears to offer benefits to the gut barrier and deserves further study.

The authors thank Xiaoyan Chang and Xiaofeng Lu for technical assistance.

DISCLOSURES

This work was supported by federal funds from the United States Department of Agriculture, Agricultural Research Service under Cooperative Agreement Number 58-6250-6-001, and from the National Institute of Child Health and Human Development [Grants HD-33920 (to D. G. Burrin) and T32-HD-07445 (to K. Kansagra)].

The contents of this publication do not necessarily reflect the views or policies of the United States Department of Agriculture, nor does mention of trade names, commercial products, or organizations imply endorsement by the United States Government.

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